Anomeric Specificity and Mechanism of Two Pentose Isomerases*

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ABSTRACT: The substrate specificities of two pentose isomerases have been determined for the aldose substrates. D-Xylose isomerase is shown to be specific for α-D-xylose or α-D-glucose. L-Arabinose isomerase uses β-L-arabinose only. Inversion of the anomeric center of the aldopyranose substrates does not seem to be catalyzed by these enzymes. The anomeric specificities of these enzymes and of phosphoglucose isomerase are discussed in terms of the formation of the *cis*-enediol intermediates that are believed to be central to their mechanisms.

Only with the specific substrate anomers can the *cis*-enediol be formed without extensive reorientation of the C_1 and C_2 hydroxyls from their cis relation in the most stable chair or boat conformers.

Extensive inquiries (Rose and O'Connell, 1961; Rose, 1962; Rose *et al.*, 1969; Dyson and Noltmann, 1968) into the mechanism of the aldose–ketose isomerases have been carried out. Generally, the open-chain (free carbonyl) forms of the sugars have been assumed to be the substrates in an enolization mechanism. This formulation, while it is reasonable and as recently indicated is correct with triose phosphate isomerase (Trentham *et al.*, 1969), cannot be true for the cases in which nonphosphorylated sugars are substrates since their rates of mutarotation at neutrality are generally too slow ($t_{1/2}^{20^\circ} \sim 6'$) and the amount of free carbonyl form present in solution is thought to be small (<2%) (Pigman and Isbell, 1968). Hines and Wolfe (1963) first suggested that phosphoglucose isomerase utilizes a cyclic form of the substrates and Salas *et al.* (1965) provided kinetic evidence that the α anomer of D-glucose 6-phosphate was the true substrate. A subsequent claim that the β anomer was the active form (Feather and Lybyer, 1969) has since been withdrawn (Feather *et al.*, 1970). Feather *et al.* (1970) have provided nuclear magnetic resonance evidence with D-xylose isomerase that the first product formed from reaction with D-xylose was the α anomer of xylose, thus suggesting that the enzyme should be specific for that form of D-xylose as substrate.

The present study inquiring into the anomeric specificity of D-xylose and L-arabinose isomerases was undertaken to test the hypothesis that the anomeric specificity of sugar isomerases is related to the occurrence of the *cis*-enediol species as an intermediate in these reactions (Rose, 1962; Rose *et al.*, 1969).

Experimental Section

Enzymes. D-Xylose isomerase was prepared from *Streptomyces* sp. by the procedure of Takasaki *et al.* (1969). L-Arabinose isomerase was prepared from *Lactobacillus brevis* according to Yamanaka (1968) as described previously (Rose *et al.*, 1969).

Sugars. α-D-Glucose (>95% α) was purchased from Baker, β-D-glucose (>95% β) and β-L-arabinose (>84% β) were obtained from Pfanstiehl Chemical Co., and α-D-xylose (>95% α) from Sigma Chemical Co. α-L-Arabinose was generously given to us by Dr. Laurens Anderson of the University of Wisconsin.

Enzyme Assay. All isomerizations were followed by assaying aliquots for ketose by the cysteine–carbazole method (Dische and Borenfreund, 1951). Standard solutions of xylulose and ribulose were derived from the fully equilibrated isomerase reaction mixtures making use of the known equilibrium constants for the isomerizations (Smyrniotis, 1962; Slein, 1962; Hochster and Watson, 1953). In these assays absorbances of 0.7, 1.03, and 1.33 are given by 0.1 μmole of fructose, xylulose, and ribulose, respectively.

Polarimetric determinations of sugar purities and mutarotation rates were made on a Schmidt and Haensch Model 10840 polarimeter.

Results

To determine the anomeric specificity of L-arabinose isomerase, initial rates were determined with fresh solutions of α- or β-L-arabinose or these same solutions after 24 mutarotation half-lives (Figure 1). The arabinose concentrations were below the K_m value found with the anomerized substrate, K_m = 33 mM (Yamanaka and Wood, 1966). As will be noted, Figure 1A, at the concentration of 9.85 mM the initial rate with β-arabinose is 2.4 times that found with the derived anomeric mixture in which only 30% or 2.95 mM is the β form. When calculated on the basis of the known K_m of the anomeric mixture and the assumption that the α anomer is neither a substrate nor an inhibitor, from which a K_m for β-L-arabinose of 10 mM may be determined, a predicted rate ratio of 2.42 is obtained. A freshly prepared solution of α-L-arabinose (4.85 mM) reacted at 55% the rate of an aged solution (Figure 1B). If the true substrate is the β anomer this rate would require that the reaction mixture contained 15% of the arabinose as the α form (the amount of substrate which would give the observed initial rate). Such a result could be explained by a small initial contamination and by

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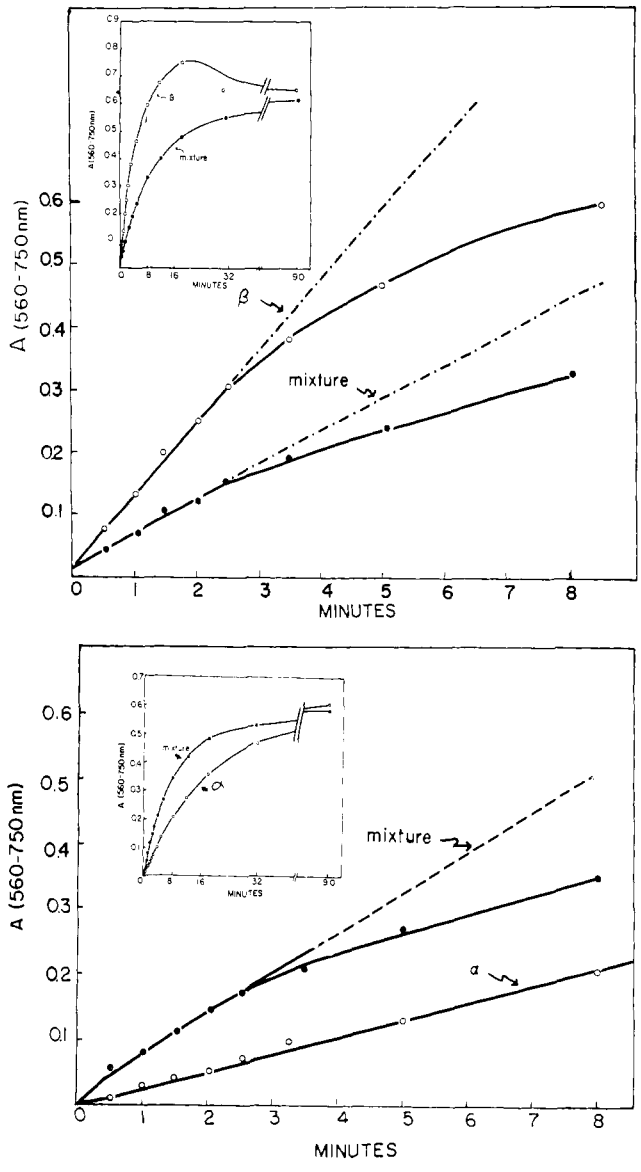


FIGURE 1: Reactions of L-arabinose isomerase utilizing α-, β-, and mutarotated L-arabinose as substrate. The following reagents were added at 26° in a final volume of 1.0 ml: 2-*N*-(morpholino)ethanesulfonic acid (MES) (pH 6.0, 50 mM), $MnCl_2$ (100 μM), L-arabinose isomerase (0.02 mg). Reaction was initiated by addition of: (A) 9.85 mM β-L-arabinose and 10 mM $CaCl_2$ (○), and 9.85 mM mutarotated L-arabinose and 10 mM $CaCl_2$ (●). Sample aliquots of 0.05 ml were analyzed; (B) 4.58 mM α-L-arabinose·$CaCl_2$·$4H_2O$ (○), and 4.58 mM mutarotated L-arabinose·$CaCl_2$·$4H_2O$ (●). Sample aliquots of 0.1 ml were taken. Similar absorbance changes in A and B are due to different assay dilution. Inserts show the reaction followed to completion.

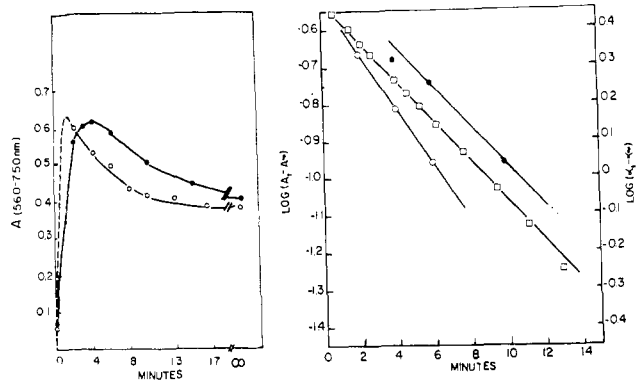


FIGURE 2: Achievement of rapid overshoot equilibrium with increasing arabinose isomerase concentration. The following reagents were added at 26°: (A) Tris-acetate (pH 7.4, 20 mM), $MnCl_2$ (200 μM), L-arabinose isomerase (0.12 mg) (○), and Tris-acetate (pH 7.4, 10 mM), $MnCl_2$ (200 μM), L-arabinose isomerase (0.06 mg) (●). Both reactions were initiated by addition of β-L-arabinose, 0.01 M, freshly dissolved. Sample aliquots of 0.025 ml were taken for color development. (B) Semilog plot of the return to final equilibrium of the data in A plus the mutarotation rate of β-L-arabinose under the latter conditions without arabinose isomerase (□).

mutarotation, which could be both spontaneous and enzyme catalyzed. The spontaneous mutarotation rate ($t_{1/2}^{25°}$ = 5.5 min) alone could maintain a concentration of β form sufficient to explain the observed rate.

In following the appearance of ketose from β-arabinose (Figure 1), an actual maximum could be observed which did not occur with either the α form or aldose equilibrium mixture. A simple explanation of this phenomenon is that the immediate equilibrium of the enzymatic isomerization of β-L-arabinose is more favorable to ketose than is the eventual overall equilibrium which includes all forms of arabinose and ribulose. If this is true, one may ask whether the progress in the readjustment to the final equilibrium state, *i.e.*, mutarotation, is enzyme catalyzed. This is related to the question of whether the enzyme catalyzes the mutarotations of ribulose, arabinose, or both. In the experiment shown in Figure 2, the rate of approach to final equilibrium at two enzyme concentrations is shown together with a semilog plot of these data and the mutarotation of β-L-arabinose measured polarimetrically in the absence of enzyme. From these data it may be concluded that if enzyme-catalyzed mutarotation occurs, its rate is less than that of the net isomerization.

Studying D-xylose isomerase substrate specificity, one is limited by the availability of only the α anomer and the mutarotated mixture of xylose. However, this enzyme also acts on D-glucose of which both anomers are obtainable. Figure 3 shows data for α- and β-glucose at 0.1 M and for the mutarotated solution which is calculated from the anomeric equilibrium (Pigman and Isbell, 1968) to contain 0.1 M α and 0.173 M β form. The initial rates were, respectively, 5.7×10^{-3}, 1.1×10^{-3}, and 7.1×10^{-3} μmole per min per mg of xylose isomerase. Only with β-glucose was a lag seen in achieving a constant rate. The simplest interpretation of these data is that the β anomer is at best a weak substrate and/or inhibitor.

Consistent with this conclusion are the data for α-D-xylose and equilibrated D-xylose shown in Figure 4. A K_m measured for the α-xylose, 3.0 mM, was 29% that measured with the anomeric mixture, 10.4 mM, which corresponds to the per cent of the mixture that is the α form (32.1–34.8%) (Pigman and Isbell, 1968). The conclusion derived from this, that any interaction of enzyme with β form must be comparatively very weak, is substantiated by the near equality of the V_{max}'s obtained by extrapolation of the initial rates.

Although this evidence suggests minimal interaction of β anomer with the enzyme, the question of whether the D-xylose isomerase liberates free aldehyde chains in the process of isomerization and thus facilitates mutarotation was investigated as with the L-arabinose isomerase by determining if the amount of enzyme influenced the rate of adjustment to the final equilibrium from that rapidly attained with the α anomer. As in the L-arabinose mixture a simple

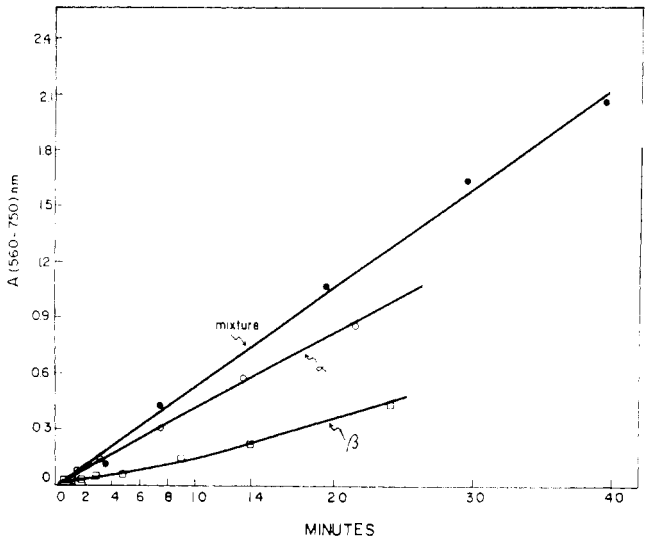


FIGURE 3: Initial velocities of D-xylose isomerase utilizing α-, β-, and mutarotated glucose as substrate. The following reagents were added at 37° in a final volume of 1.0 ml: MES (pH 6.0, 50 mM), $MnCl_2$ (1 mM), $CoCl_2$ (1 mM), D-xylose isomerase (1.05 mg). Reaction was initiated by addition of 0.099 M α-glucose (○), 0.097 M β-glucose (□) (both freshly dissolved), and 0.27 M mutarotated glucose (●). Sample aliquots of 0.1 ml were taken.

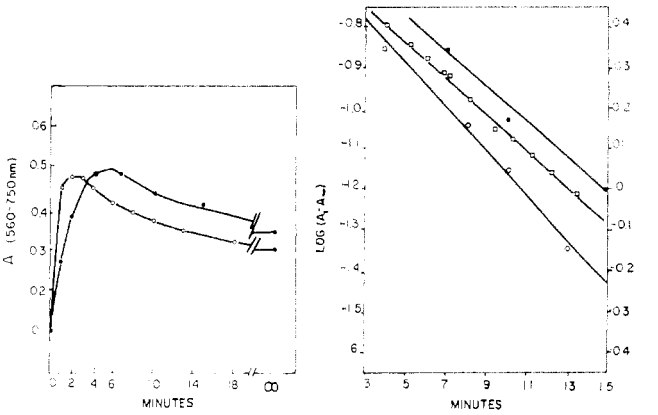


FIGURE 5: Achievement of rapid overshoot equilibrium with increasing xylose isomerase concentration. The following reagents were added at 26°: (A) Tris-maleate (pH 7.0, 2 mM), $MgCl_2$ (5 mM), $CoCl_2$ (5 mM), D-xylose isomerase (3.48 mg) (○) (sample aliquots of 0.02 ml were used) and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (pH 7.4, 50 mM), $CoCl_2$ (2 mM), D-xylose isomerase (0.9 mg) (●) (sample aliquots of 0.05 ml were taken for color development. Reactions were initiated by 7 mM α-D-xylose (○), and 2.7 mM α-D-xylose (●), freshly dissolved. Differing assay dilutions result in similar absorbance changes. (B) Semilog plot of the return to final equilibrium of the data in A plus the mutarotation rate of α-D-xylose under the conditions of the pH 7.0 experiment without xylose isomerase (●).

first order approach to equilibrium is observed with the anomeric mixture of D-xylose. As noted in Figure 5, a pronounced overshoot was again observed and the semilog plot of the approach to final equilibrium indicates little catalysis beyond the nonenzymatic rate. A rate of mutarotation 20% that of isomerization would have been observable.

Three possible cases may be distinguished concerning the processes that might be occurring in the adjustment to the final equilibrium: (1) an enzyme-catalyzed aldose anomeric interconversion coupled with nonenzymatic ketose anomerization; (2) an enzyme-catalyzed ketose anomerization and nonenzymatic aldose mutarotation; (3) neither mutarotation is enzyme catalyzed. Case 1 would produce a metastable equilibrium between the two aldoses and one ketose. The final equilibrium would shift to include a population of the other anomeric ketose as well and would thus give rise to more ketose and the observation of an undershoot. Case 2 would initially give both ketoses and one aldose and subsequently shift to less ketose. This would be an overshoot. From the known values of the anomeric aldose equilibria the overshoot ratio may be calculated to be 2.3 and 2.6 for xylose and arabinose, respectively. These values are greater than those observed which, plotting the value of maximum ketose concentration reached *vs.* the time that the maximum was achieved, extrapolate to 1.9 and 2.0, respectively, at time zero, *i.e.*, infinite enzyme concentration. The results are consistent with case 3, however, and allow one to calculate, in each case, the portion of the equilibrium mixture of the ketopentose anomers that is the enzymatically formed product, as follows:

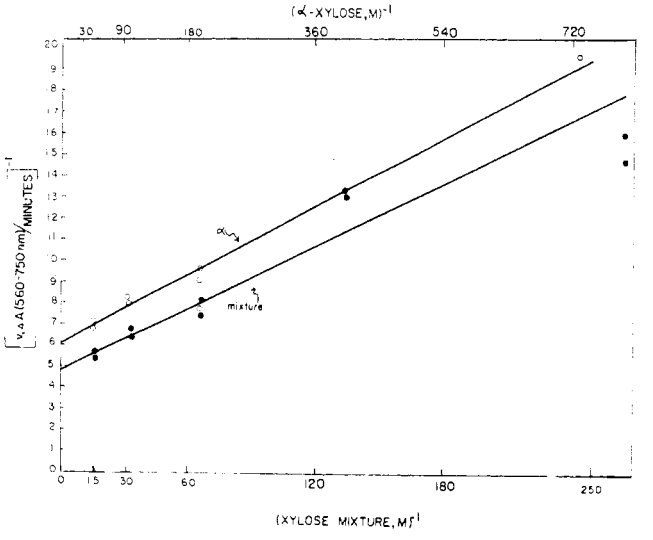


FIGURE 4: Effect of α-D-xylose and mutarotated D-xylose concentration on xylose isomerase. The following reagents were added at 26° in a final volume of 1.0 ml: MES (pH 6.0, 50 mM), $MnCl_2$ (5 mM), D-xylose isomerase (0.48 mg). Sample aliquots of 0.1 ml were taken. The solid line was obtained utilizing program 1047A/GS2 of the Wang 700 series calculator.

Let C_e, C_n, A_e, and A_n be the concentrations of the ketoses, C, and aldoses, A, that are either enzymatically active or not, e or n. Let K_e be the metastable equilibrium constant, $K_e = C_e/A_e$, and let K_f be the final equilibrium constant, $K_f = (C_e + C_n)/(A_e + A_n)$. K_c and K_a are the equilibrium ratios C_n/C_e and A_n/A_e, respectively. Rearranging the expression for K_f gives

$$K_f = C_e/A_e \frac{(1 + C_n/C_e)}{1 + A_n/A_e} \quad (1)$$

Substituting the terms defined above gives

$$\frac{K_f}{K_e} = \frac{1 + K_c}{1 + K_a} \quad (2)$$

TABLE I: Comparative Anomeric Specificities of Several Isomerases.

Enzyme	"Preferred" Anomer Aldose	OH Attitudes 1	OH Attitudes 2	References
Phosphoglucose isomerase	α-D-Glucose-6-P	a	e	Salas *et al.* (1965)
D-Xylose isomerase	α-D-Glucose	a	e	This work
D-Xylose isomerase	α-D-Xylose	a	e	This work; Feather *et al.* (1970)
L-Arabinose isomerase	β-L-Arabinose	a	e	This work

which rearranges to

$$K_e = \frac{K_f}{K_e}(1 + K_a) - 1 \qquad (3)$$

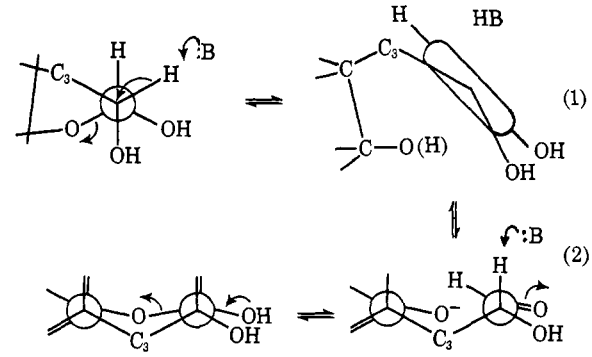
The ratio of the peak ketose concentration to that present at final equilibrium was determined by extrapolation of the data of Figures 2 and 5 to infinite enzyme concentrations. From this value and the reported values of K_f one calculates K_e. Using the known values of K_a one obtains K_c of 0.72 and 0.23 for arabinose and xylose isomerases, respectively. Hence, the L-ribulose anomer formed in the L-arabinose isomerase reaction is slightly dominant, 58%, among the equilibrium ribulose forms and the enzymatically formed D-xylulose anomer is greatly dominant, 81%, among the products of the xylose isomerase reaction. Unfortunately it is not known which form is dominant among the anomeric ketopentoses so that the structural assignment of the enzyme products is not possible at this time.

Discussion

The evidence cited suggests that the pentose isomerases show specificities for one of the anomeric aldose forms. The relative upper limit for the rate of reaction of L-arabinose isomerase with the unfavored α-arabinose is calculated to be about 20% at 10 mM. In the case of D-xylose isomerase the maximum reactivity that might be attributable to β-glucose is also about 20% of the rate with α-glucose. These represent the upper limits derived from initial rate studies. The fact that the K_m's of α-xylose and the mutarotated mixture are related as expected if only the α anomer is bound and that the V_{max} of the mixture is not less than that for α-xylose is further strong evidence for only a negligible interaction of xylose isomerase with the β anomer. From the height of the overshoot it may be concluded that both enzymes catalyze only the interconversion of particular aldose and ketose forms.

The dispositions of hydroxyl at C_1 and C_2 of the most stable conformers (Reeves, 1950) of the aldose anomers found to be substrates of the isomerases are shown in Table I. In the four cases studied, representing three enzymes, the C_1 and C_2 hydroxyls are relatively cis. In catalyzing the isomerization reaction it is evident that the bonds from C_1 to the ring oxygen as well as that from C_2 to its hydrogen must open. One may reach the *cis*-enediol proposed to be an intermediate in these reactions by two basically different processes: (1) direct elimination of the C_2 proton and the C_1 ring oxygen, or (2) ring opening followed by enolization (Scheme I). One can rationalize the anomeric specificities in terms of an elimination mechanism since only anomers with the *cis*-1,2-hydroxyl configuration are capable of undergoing an anti

SCHEME I

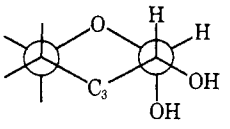


elimination to form a *cis*-enediol. This can occur only from the skew boat conformation. An anti elimination from the other anomer yields a *trans*-enediol. Thus, a concerted elimination mechanism for producing a *cis*-enediol would explain the anomeric specificity. Possible evidence against this mechanism is the observation that α-methyl xylopyranoside at concentrations that nuclear magnetic resonance data show to give good binding (K. J. Schray and A. S. Mildvan, unpublished) was not converted into a ketose when incubated with enzyme for several days.

Mechanism 2 (Scheme I) accommodates ring opening to the classical enolization mechanism, making use of the carbonyl group to delocalize the charges that develop during proton abstraction. According to mechanism 2, ring closure inevitably leads to a cis relation between the C_1 and C_2 hydroxyls of the aldose if the cis relation of the enediol is maintained in the open chain form of the enzyme-bound aldose, that is, if rotation at the C_1–C_2 axis is prevented at the time of ring closure. Proton addition is believed to occur at C_2 and C_1 from the same face of the *cis*-enediol, as shown by the occurrence of intramolecular proton transfer with the required stereospecificity (Rose, 1962; Rose *et al.*, 1969). When the proton adds to C_2 the carbon chain beyond C_2 must become oriented so that the C_5 hydroxyl can approach the carbonyl C_1 only from the opposite direction of the original proton approach to C_2. Looking at the reaction in the forward direction, ring opening would not occur unless the C_1 and C_2 hydroxyls assume the cis-eclipsed conformation required mechanistically. This relationship can occur only in the boat form of the substrate anomer. This mechanism thus also accounts for the selective anomeric specificity. A model for such a selective interaction exists in the observed selective chelation by borate of sugars with *cis*-hydroxyls (Boseken, 1949).

On the basis of these considerations one would predict for other isomerases that where anomeric specificity is manifest it will favor the form in which the anomeric hydroxyl of the aldose is in a cis relation with the C_2 hydroxyl.

Alternatively, the specificity may be viewed as due simply to the stereochemistry of the isomerase binding site. All of the examined anomers have as the most stable conformation, I. According to this alternative hypothesis, in the evolution



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of the isomerases of different sugar specificities the nature of the substrate binding site was altered to accommodate the different substrates. Their specificity for the anomeric positions, however, was conserved for reasons not related to reaction mechanism but perhaps to genetic or structural factors. Examples of the occurrence of this cis-anomeric specificity for other aldose isomerases are required before this alternative can be ruled out.

Both proposed mechanisms utilize the low percentage of substrate existing in the boat conformation. This apparent disadvantage may be overcome by enzymic catalysis of the chair–boat interconversion and/or utilization of the higher energy of the boat conformation for progress toward the transition state. This correlation of anomeric substrate specificity with the geometry of the enediol suggests that some of the same groups on the enzyme, acids, bases, and metal ion, may function in determining both stereospecificities.

The two isomerases investigated here appear not to catalyze the mutarotation of their substrates in contrast to phosphoglucose isomerase (Salas *et al.*, 1965) (although the mutarotase assay in these cases is not as sensitive as in the latter). Thus, open-chain intermediates, if formed, would have to remain tightly bound.

In conclusion, L-arabinose isomerase has been shown to utilize specifically the β-L-arabinopyranose, and generation by D-xylose isomerase of α-D-xylopyranose reported earlier has been substantiated and extended to α-D-glucopyranose. These anomers and the α-D-glucose 6-phosphate previously reported to be utilized by phosphoglucose isomerase all bear the C_1–C_2 hydroxyls in a cis relationship, which has been interpreted mechanistically. The pentose isomerases seem to show little mutarotase activity.

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